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ORIGINAL PAPER

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Constitutive expression of the *Trichoderma reesei* β-1,4-xylanase gene (*xyn2*) and the β-1,4-endoglucanase gene (*egI*) in *Aspergillus niger* in molasses and defined glucose media

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Abstract The xylanase II (*xyn2*)- and endoglucanase I (*egI*)-encoding regions of *Trichoderma reesei* QM6a were successfully expressed in *Aspergillus niger* D15 under the transcriptional control of the glyceraldehyde-6-phosphate dehydrogenase (*gpd*) promoter from *A. niger* and the *glaA* terminator of *Aspergillus awamori*. A stable *xyn2* transformant produced β-xylanase activity of 8,000 nkat/ml and 5,000 nkat/ml in shake-flask cultures containing defined or 20% (v/v) molasses medium, respectively. The recombinant Xyn2 enzyme expressed highest activity at pH 5–6 and 50–60 °C and retained more than 75% of its activity after 3 h of incubation at 50 °C. A stable *egI* transformant produced endo-β-1,4-glucanase activity of 2,300 nkat/ml in shake-flask cultures containing defined media and about half the activity in 20% molasses medium. Maximum endoglucanase activity was obtained at pH 5 and 60 °C. Both Xyn2 and EgI retained >80% activity after incubation at 50 °C for 3 h. The heterologous Xyn2 and EgI represent a significant portion of the total extracellular proteins produced.

Introduction

Plant material consists mainly of cellulose, hemicellulose and lignin. Cellulose represents the largest form of fixed carbon in nature. The cellulose strand consists of β-1,4-linked glucopyranose units (Béguin and Aubert 1994). Each glucose molecule is rotated at 180° relative to it's neighbor, making cellobiose the basic repeating unit. These molecules form inter- and intramolecular hydrogen-bonding patterns which account for the rigid, insoluble microfibrils. Endo-β-1,4-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) are required for the degradation of cellulose to glucose (Aristidou and Penttilä 2000).

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Hemicelluloses are low molecular weight heteropolysaccharides with a wide variation in both structure and composition. Commonly occurring hemicellulases include the 1,3- and 1,4-β-galactans, mannans and xylans (Aristidou and Penttilä 2000). Xylan is the main form of hemicelluloses present in plant cell walls and can constitute up to 35% of the dry weight of plants (Puls and Schuseil 1993). The xylan structures vary from linear β-1,4-polyxylose main chains to highly branched polysaccharides containing acetyl, arabinosyl and glucuronosyl side chains. Endo-β-1,4-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) work synergistically to degrade xylan to xylose. α-Arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139) and acetyl xylan esterases (EC 3.2.1.72) are responsible for the debranching of the xylan backbone.

Cellulases and hemicellulases have a broad spectrum of industrial applications including the production of fuel ethanol (using agricultural waste products), the pulp and paper industry (reducing the amount of chlorine required for bleaching), the baking industry (increasing the volume of dough), the animal feed industry (improving digestion and production efficiency), the wine and fruit juice industry (clarification of juice), etc. (Aristidou and Pentilä 2000; Colagrande et al. 1994; Oksanen et al. 2000). Therefore, a large potential market exists for cellulases and hemicellulases.

The advantages of using *Aspergillus* spp. as host for heterologous expression include its high secretion capacity, GRAS (*g*enerally *r*egarded *a*s *s*afe) status, rapid growth on inexpensive media and a relatively well-studied genetic background. *Aspergillus* spp. has the ability to produce heterologous proteins in concentrations of grams per liter (Verdoes et al. 1995) and has been used by various industries for the production of a diversity of commodities such as citric and gluconic acid, enzymes such as proteases, catalases, isomerases, α-galactosidases, rennin, lipase, phytase, glucoamylase, pectinase, glucose oxidase, α-amylase, as well as pharmaceuticals such as interleukin-6 and Fab (Archer 2000; Gibbs et al. 2000). World sales of β-lactam antibiotics produced by

Aspergillus spp. have been estimated at US$ 9 billion for 1997 alone (Gibbs et al. 2000).

Aspergillus spp. produce high levels of β-glucosidase activity, but have weak levels of endoglucanase activity (Duff et al. 1986). The cellulolytic fungus *Trichoderma reesei*, on the other hand, exhibits potent endoglucanase activity but limited β-glucosidase activity (Duff et al. 1986; Ghose et al. 1985; Takashima et al. 1999). The endoglucanase EgI of *T. reesei* is of particular interest due to its ability to hydrolyze a wide range of substrates such as avicel, carboxymethyl cellulose (CMC), hydroxyethyl cellulose (HEC), barley β-glucan, acid-swollen amorphous cellulose, lichenan, xylan and even galactomannan (Bailey et al. 1993b). However, the β-glucosidases of *T. reesei* account for only 1% of the total protein secreted and this insufficient activity is known to limit the saccharification of cellulose (Takashima et al. 1999). Therefore, the *Aspergillus* and *Trichoderma* genera have been used in mixed culture with great success since their cellulolytic enzyme systems compliment each other (Chadha and Garcha 1992; Duff et al. 1986; Friedrich et al. 1987; Ghose et al. 1985; Maheshwari et al. 1994). Enzyme production by mixed cultivation, however, is inherently difficult to optimize since the organisms require different growth conditions, media, etc. The production of cellulase enzyme cocktails from separate fungal hosts is also costly. This motivated us to evaluate recombinant *A. niger* strains capable of constitutively producing enzymes of *T. reesei* while using molasses, an industrial waste product of the sugar industry, as carbon source.

Here, we describe the construction of a constitutive fungal expression cassette consisting of the glyceraldehyde-3-phosphate dehydrogenase promoter (gpd_p) of *A. niger* and the glucoamylase terminator ($glaA_T$) of *Aspergillus awamori*. The *xyn2*- and *egI*-encoding regions of *T. reesei* were subsequently constitutively expressed in *A. niger* under the transcriptional control of the *gpd* promoter sequences and the production of the recombinant enzymes in a simple industrial medium (molasses) compared to the production in a defined medium. The enzymatic properties of the recombinant Xyn2 and EgI were also characterized.

Material and methods

Media and cultivation conditions

Recombinant plasmids were constructed and amplified in *Escherichia coli* DH5α and cultivated at 37 °C in Terrific Broth and on Luria Bertani agar containing 100 µg ampicillin/ml for selective pressure (Sambrook et al. 1989). Fungal strains were cultivated at 30 °C in 20% (v/v) molasses (Tongaat-Huletts, South Africa) or minimal media containing 0.5% (w/v) yeast extract, 0.2% (w/v) casamino acids, 1% (w/v) glucose, 6% $NaNO_3$ (w/v), trace elements and 0.01 M uridine (Punt and van den Hondel 1992). Transformants were selected on minimal medium containing 10 mM acetamide and 15 mM CsCl, but lacking $NaNO_3$. Media were inoculated to a concentration of 1×10^5 spores per ml unless stated otherwise. *A. niger* D15 transformants were cultivated in 20% molasses solution and in double-strength minimal media (2xMM) containing 10% glucose for enzyme activity determination.

Strains and plasmids

The genotypes of the bacterial and fungal strains as well as the plasmids used in this study are summarized in Table 1.

DNA manipulations and amplification by PCR

Standard protocols were followed for all DNA manipulations and *E. coli* transformations (Sambrook et al. 1989). *A. niger* was transformed by means of spheroplasts using Lyzing enzymes (Sigma) in accordance with Punt and van den Hondel (1992).

Table 1 The genotype and sources of the strains and plasmids used in this study

	Genotype	Source
Strains:		
E. coli DH5α	*supE44 ΔlacU169 (Ø80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Sambrook et al. (1989)
A. niger van Tieghem	Wild-type	ATCC 10864
A. niger D15	*pyrG prtT phmA* (nonacidifying)	Wiebe et al. (2001)
A. niger D15[pGT]	*A. niger* D15 with gpd_P-$glaA_T$ integrated into the chromosome	This study
A. niger D15[*xyn2*]	*A. niger* D15 with gpd_P-*xyn2*-$glaA_T$ integrated into the chromosome	This study
A. niger D15[*egI*]	*A. niger* D15 with gpd_P-*egI*-$glaA_T$ integrated into the chromosome	This study
T. reesei QM6a	Wild-type	ATCC 13631
Plasmids:		
pSPORT1	*bla*	Gibco BRL Life Technologies
pKSExtrendedglAPo	*bla* $glaA_T$	Stewart et al. (1996)
pDLG5	*bla URA3* $ADH2_P$-*xyn2*-$ADH2_T$	La Grange et al. (1996)
p3SR2	*bla amdS*	Wernars et al. (1985)
pSPORT-$glaA_T$	*bla* $glaA_T$	This study
pGT	*bla* gpd_P-$glaA_T$	This study
pGT-xyn2	*bla* gpd_P-*xyn2*-$glaA_T$	This study
pSPORT-egI	*bla egI*	This study
pGT-egI	*bla* gpd_P-*egI*-$glaA_T$	This study
pSPORT-gpd_p	*bla* gpd_P	This study
pSPORT-xyn2	*bla xyn2*	This study

Table 2 The DNA sequences and sizes of the oligodeoxyribonucleotide primers used in this study. Restriction sites are in *italics*

Primer name	Sequence[a]	Primer size	Restriction enzyme sites
GPDR	5′ GTAC*GCGGCCGC*TGTTTAGATGTGTCTATGTGGC 3′	34	*Not*I
GPDL	5′ GATC*GGATCC*GAGCATCACCAACATGGTAC 3′	30	*Bam*HI
TregR	5′ *GCGGCCGCAGATCT*AGTCAACGCTCTAAAGGCA 3′	33	*Not*I-*Bgl*II
TregL	5′ *GCGGCCGCGAATTC*AATGGCGCCCTCAGTTA 3′	30	*Not*I-*Eco*RI
EgverR	5′ CTAAAGGCATTGCGAGTAGTAGTCGTTGCTATACTGGCAC 3′	40	
EgintrR	5′ TATCTCCGGGGCCGTAGTAGCTTTTGTAGCCGCTGCC 3′	37	

Fungal strains were grown in minimal media for 72 h. Mycelia were harvested and frozen under liquid nitrogen and DNA isolated according to La Grange et al. (1996). The *gpd* promoter (gpd_P) was amplified from the genome of *A. niger* ATCC10864 as a 1.2-kb fragment, using PCR and oligonucleotide primers GPDR and GPDL (Table 2). PWO polymerase (Roche) was used for amplification with the reaction set up in accordance with the supplier's specifications. The genomic copy of *egI* was amplified from the genome of *T. reesei* QM6a using primers TregR and TregL. The 1.6-kb genomic copy of *egI* was used as template to produce the cDNA via overlapping PCR, using primers TregL, EgverR (with the first 20 nucleotides being homologous to the entire third exon and the last 20 nucleotides being homologous to the 3′-end of the second exon) and EgintrR (with the first 20 nucleotides being homologous to the 5′-end of exon 2 and the last 17 nucleotides homologous to the 3′-end of exon 1). The standard PCR reaction yielded a 1.4-kb fragment containing the coding region of *egI* without introns, but with the secretion signal still intact.

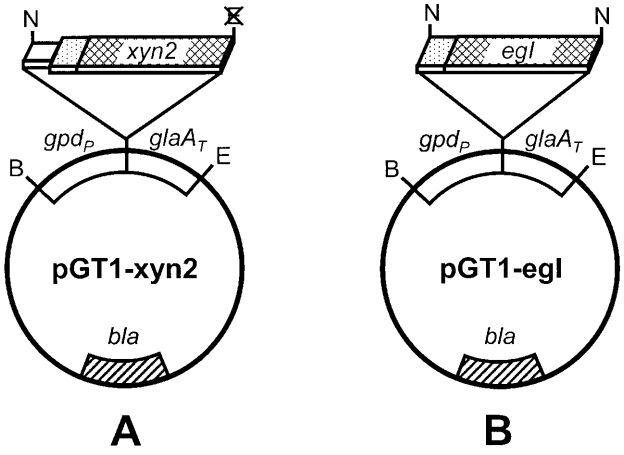

Fig. 1 Expression vectors **A** pGT-xyn2 and **B** pGT-egI. The *xyn2* and *egI* genes are indicated with *cross-hatched boxes*, their secretion moieties with *dotted boxes*, the selectable marker (*bla*) by a *hatched box*. The *gpd* promoter and terminator sequences are indicated by the *open boxes* and the pSPORT1 sequences are indicated by *thick lines*. The restriction sites indicated are *Bam*HI (*B*), *Eco*RI (*E*) and *Not*I (*N*)

Plasmid construction

The *glaA* terminator from *A. awamori* was amplified from plasmid pKSExtendedglAPo (Stewart et al. 1996) as an 1.0-kb *Eco*RI-*Sal*I fragment and cloned into the corresponding sites on pSPORT1, generating pSPORT-glaA$_T$. The amplified 1.2-kb gpd_P PCR fragment was cloned into pSPORT1 digested with *Sma*I, resulting in pSPORT-gpd$_P$. This plasmid was used to confirm the DNA sequence of the gpd_P DNA fragment. The gpd_P was subsequently retrieved by digesting pSPORT-gpd$_P$ with *Bam*HI and *Not*I and cloned into the corresponding sites in pSPORT-glaA$_T$, generating plasmid pGT. Plasmid pGT formed the expression vector used for the constitutive expression of recombinant genes in *A. niger* D15.

The *xyn2* gene (cDNA including the native secretion signal) was derived from pDLG5 (La Grange et al. 1996) by digestion with *Eco*RI and *Bgl*II. The 5′ overhanging end was filled in by the addition of Klenow polymerase (Roche) and cloned into the *Sma*I site of pSPORT1. The *xyn2* gene was subsequently retrieved by digestion with *Eco*RI (subsequently filled in by using Klenow) and *Not*I, and cloned into pGT digested with *Sal*I (site filled in by using Klenow) and *Not*I, creating pGT-xyn2 (Fig. 1A).

The PCR product containing the *egI* open reading frame (ORF) was cloned into pSPORT1 digested with *Sma*I, resulting in plasmid pSPORT-egI. This plasmid was used to confirm the DNA sequence of the *egI* gene. The *egI* was retrieved by digestion with *Not*I and the 1.4-kb DNA fragment cloned into the corresponding site in pGT, resulting in pGT-egI (Fig. 1B).

DNA hybridizations

Southern hybridizations were carried out according to Sambrook et al. (1989). The internal 540-bp *Eco*RI–*Xho*I fragment of *xyn2* and the internal 685-bp *Eco*RI–*Xho*I fragment of *egI* were labeled with [α-^{32}P]ATP using the Random Primed Kit (Roche), according to the supplier's specification. The number of integrations was determined by digesting the genome of both the *xyn2* and *egI* transformants overnight with *Hin*dIII. DNA were separated on a 0.8% agarose gel and used for traditional Southern blot analysis.

Enzymatic assays

Transformants were screened on RBB-xylan and OBR-HECellulose plates containing 10% glucose for inhibition of the native xylanases and cellulases. Xylanase and endoglucanase activity, as well as pH and temperature optima determination of Xyn2 and EgI, was done as described by Bailey et al. (1992), using citrate phosphate buffer (0.05 M). The substrates used for liquid assays were 1% birchwood xylan (Roth), 1% CMC (Sigma) and 0.1% lichenan (Sigma) resuspended in 0.05 M sodium citrate buffer (pH 5 for Xyn2 and pH 5.7 for EgI). All enzymatic assays were done in triplicate. One unit of enzyme was defined as the activity producing 1 µmol per min of reducing sugars in glucose or xylose equivalents under these assay conditions.

Protein isolation and gel electrophoresis

The supernatants produced by the different transformants were collected after 3 days of growth and freeze-dried before determining the specific activity. Two mg of freeze-dried supernatant (containing about 50 µg of total extracellular protein) was separated by 10% (in the case of the EgI) and 15% (in the case of Xyn2) SDS-PAGE using the Gibco Protein marker (Gibco) to estimate the size of the protein. The protein gel was stained with Coomassie brilliant blue and destained in accordance with Ausubel et al. (1998). Protein concentrations were determined with the aid of the Biorad Protein Assay.

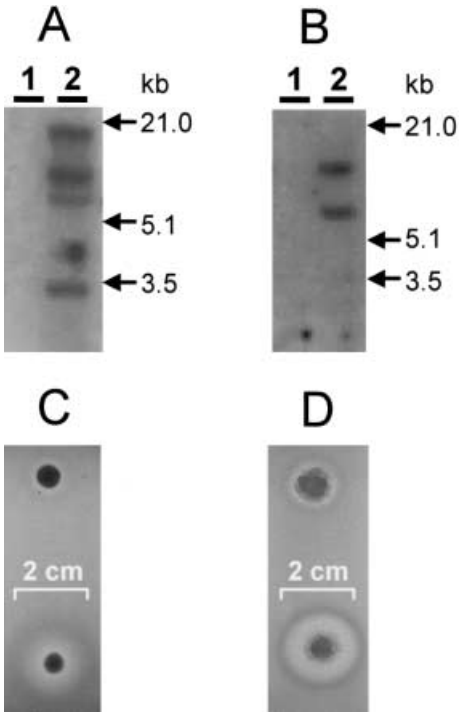

Fig. 2 Southern blot analysis to determine the copy number of the *xyn2* and *egI* genes present on the genome of **A** *A. niger* D15[*xyn2*] and **B** *A. niger* D15[*egI*]. Total DNA was isolated and digested overnight with *Hin*dIII. *Lane 1* DNA isolated from *A. niger* D15[pGT], *lane 2* (**A**, **B**) DNA isolated from *A. niger* D15[*xyn2*] and *A. niger* D15[*egI*]. The internal 540-bp *Eco*RI–*Xho*I fragment of *xyn2* and the internal 685-bp *Eco*RI–*Xho*I fragment of *egI* were labeled with [α-^{32}P]ATP and used as DNA probes. Marker sizes are indicated on the *right*. Each ^{32}P-highlighted DNA species represents a copy of the *xyn2* or *egI* gene. **C** Recombinant xylanase produced by *A. niger* D15[*xyn2*] on RBB-xylan plates; **D** recombinant endoglucanase activity produced by the *A. niger* D15[*egI*] on OBR-HECellulose plates. The negative control, *A. niger* D15[pGT], is shown at the *top* with the transformant at the *bottom*. Colonies degrading RBB- or OBR-HECellulose are surrounded by pale clearing zones. The plates were photographed after 48 h incubation at 30 °C

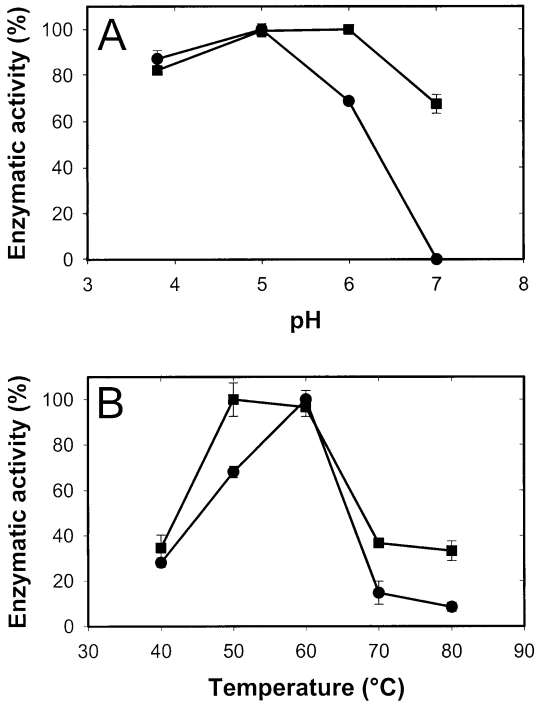

Fig. 3 The effect of **A** pH and **B** temperature on the enzymatic activity of the Xyn2 (■) and EgI (●) when produced by *A. niger* D15. The highest activity was measured at pH 5.5 and 55 °C for the Xyn2, and pH 5 and 60 °C for the EgI

Results

Construction of recombinant *A. niger* strains containing *xyn2* and *egI* gene copies

Aspergillus niger D15, a pH mutant strain, was used as host for the heterologous production of the *T. reesei* xylanase II and endoglucanase I enzymes in molasses and defined glucose media. Plasmids pGT, pGT-egI and pGT-xyn2 were co-transformed with plasmid p3SR2 into *A. niger* D15, and the expression cassettes integrated into the *A. niger* genome in multiple sites. Plasmid p3SR2 contained the *amd*S gene which allowed for selection of *A. niger* transformants capable of acetamide and acrylamide utilization in the absence of other nitrogen sources (Wernars et al. 1985). The transformant containing pGT (designated *A. niger* D15[pGT]) acted as negative control.

Chromosomal DNA was isolated from *A. niger* D15[pGT] as well as the *xyn2* and *egI* transformants (designated *A. niger* D15[*xyn2*] and *A. niger* D15[*egI*], respectively) that expressed the highest level of recombinant enzyme activity. The DNA was digested overnight with *Hin*dIII, a restriction enzyme that does not cut within the *xyn2* or *egI* coding regions, and used for Southern blot analysis. Southern blot analysis of the digested chromosomal DNA showed that multiple copies of the genes had integrated into the genomes of the transformants. At least five copies of the *xyn2* gene were determined to be present in the genome of *A. niger* D15[*xyn2*]. The native *A. niger* xylanase genes were not detected (Fig. 2A). At least two copies of the *egI* gene were present in the *A. niger* D15[*egI*] (Fig. 2B).

Plate assays for enzymatic activity

Spores of *A. niger* D15[*xyn2*] as well as those of the control strain, *A. niger* D15[pGT], were spotted onto RBB-xylan activity plates containing 10% (w/v) glucose. *A. niger* D15[*xyn2*] produced a zone after about 6 h of growth, whereas, *A. niger* D15[pGT] only produced a zone after 48 h, upon depletion of the glucose surrounding the colony (Fig. 2C). *A. niger* D15[*egI*] started producing a clearing zone on OBR-HECellulose after 10 h, whereas *A. niger* D15[pGT] only started to produce a zone after about 72 h of growth (Fig. 2D).

Table 3 Comparison of the characteristics of Xyn2 and EgI when produced by different hosts. *nd* Not determined

	T. reesei	*A. niger* D15	*Y. lipolytica*	*S. cerevisiae*
Xyn2:				
Enzyme activity (nkat/ml)	5400 [Bailey et al. (1993a)]	8100		1200 [La Grange et al. (1996)]
pH optimum	5–5.5 [Törrönen and Rouvinen (1995)]	5–6		6 [La Grange et al. (1996)]
Temperature optimum (°C)	56–60[a] [Dekker (1983)]	50–60		60 [La Grange et al. (1996)]
Protein size (kDa)	20 [Lappalainen et al. (2000)]	21		27 [La Grange et al. (1996)]
EgI:				
Enzyme activity (nkat/ml) on CMC	1469.6 [Montenecourt (1983)[b]]	2300	367.4 [22 U/ml Park et al. (2000)]	119[c]
pH optimum	4–5 [Takashima et al. (1998), Zurbriggen et al. (1991)]	5 [4[d] in Takashima et al. (1998)]	4.8 [Park et al. (2000)]	6 [Zurbriggen et al. (1991)]
Temperature optimum (°C)	60 [Zurbriggen et al. (1991]	60	50 [Park et al. (2000)]	60 [Zurbriggen et al. (1991)]
Protein size (kDa)	50 [Takashima et al. (1998), Kleywegt et al. (1997)]	62–100 [67[d] in Takashima et al. (1998)]	60–80 [Park et al. (2000)]	60–100 [Takashima et al. (1998)]

[a] The combined action of all the xylanases produced by *T. reesei*
[b] The cellulolytic mutant RUT C-30 can produce levels of up to 225 IU/ml [3757.5 nkat/ml, Montenecourt (1983)]
[c] Values obtained in this study using a multicopy episomal plasmid and the *ADH2* promoter for expression in SC^{-URA} medium (La Grange et al. 1996)
[d] Expression in *Aspergillus oryzae* as host

Effect of pH and temperature on xylanase activity

The optimum levels of activity for the recombinant Xyn2 xylanase were achieved between pH 5 and 6 and at a temperature of 50–60 °C (Fig. 3A). This is similar to the pH and temperature optima of recombinant Xyn2 produced by *Saccharomyces cerevisiae* (La Grange et al. 1996) as well as the native Xyn2 produced by *T. reesei* (Törrönen and Rouvinen 1995). Table 3 summarizes the characteristics of the native and heterologously produced Xyn2. The recombinant EgI endoglucanase produced by *A. niger* exhibited its highest levels of activity at pH 5 and 60 °C (Fig. 3B).

The thermostability of recombinant Xyn2 and EgI produced by *A. niger* was determined over a period of 3 h (Fig. 4). It was found that both the Xyn2 and the EgI enzymes still retained more than 80% of their activity after 3 h of incubation at 50 °C, but lost almost all activity within 2 h at 70 °C. However, EgI retained about 50% activity when incubated at 60 °C for 3 h.

Heterologous enzyme production

The production of heterologous Xyn2 and EgI by *A. niger* D15[*xyn2*] and *A. niger* D15[*egI*], respectively, was followed over a time period of 15 days in molasses as well as in 2xMM. The highest level of activity for Xyn2 was achieved on day 9 in molasses and day 10 in 2xMM medium (Fig. 5). The highest level of activity obtained with *A. niger* D15[*xyn2*] was 8,000 nkat/ml, which is about six times more than that obtained with expression in *S. cerevisiae* and 1.5 times more than that obtained in

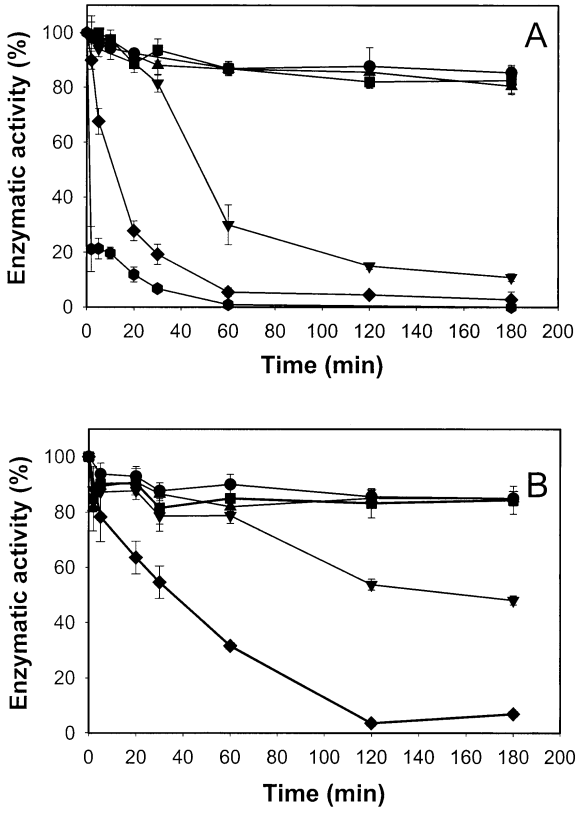

Fig. 4 The thermostability of **A** recombinant Xyn2 and **B** EgI produced by *A. niger* D15 at 4 °C (●), 40 °C (■), 50 °C (▲), 60 °C (▼), 70 °C (◆) and 80 °C (*filled hexagon*). Freeze-dried enzyme (0.2 µg) was pre-incubated at the various temperatures in the absence of the substrate for up to 180 min before the remaining activity was determined. The activity determined prior to the incubations was taken as 100%

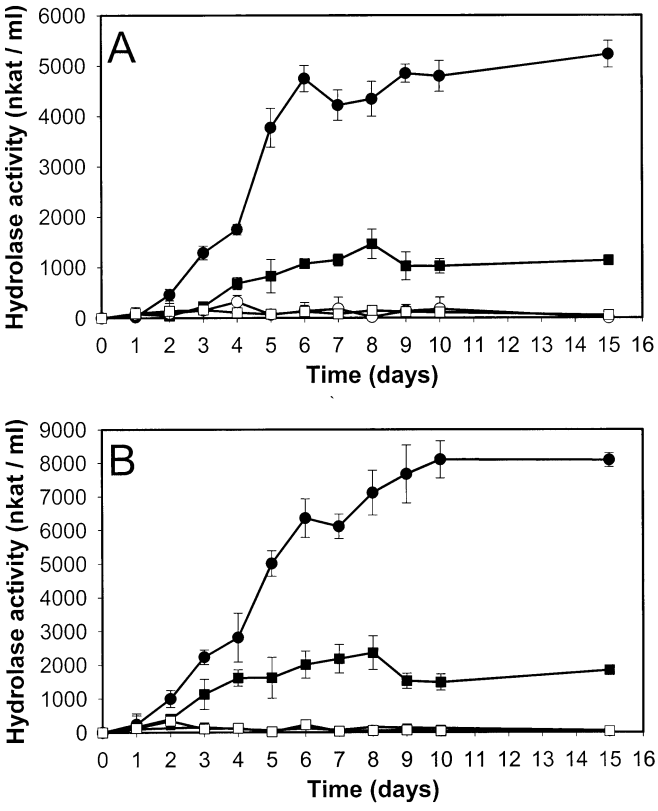

Fig. 5 The heterologous production of xylanase (*circles*) and endoglucanase (*squares*) by *A. niger* D15[*xyn2*] (●) and *A. niger* D15[*egI*] (■) and *A. niger* D15[pGT] (○, □)was monitored over a period of 15 days in **A** molasses and **B** 2xMM medium. The pH of the media did not change significantly. The pH dropped from 5.5 to 4.5 after 3 days of cultivation and remained at pH 4.5 for the duration of the experiment. Enzyme activity assays were determined in triplicate using six parallel cultures. Enzyme activity was determined as described in La Grange et al. (1996)

T. reesei (La Grange et al. 1996; Bailey et al. 1993a). The highest level of xylanase activity reached with growth in molasses was 5,000 nkat/ml. At day 8, *A. niger* D15[*egI*] produced endoglucanase activity levels of 1,400 and 2,300 nkat/ml in molasses and 2xMM, respectively.

Heterologous protein isolation and SDS-PAGE analysis

The supernatant of *A. niger* D15[pGT], *A. niger* D15[*xyn2*] and *A. niger* D15[*egI*] was harvested on day 4 and freeze-dried in order to concentrate the enzymes produced in 2xMM. In all cases the total extracellular protein content was determined as about 4% and the mixture was used without further purification. The activity of the recombinant Xyn2 in the unpurified supernatant was determined as 54 nkat/mg. The activity of the recombinant EgI was determined as 11.1 and 48 nkat/mg in the unpurified supernatant on CMC and lichenan as substrates, whereas *A. niger* D15[pGT] yielded only 11.8 and 1.3 nkat/mg of unpurified supernatant on CMC and lichenan, respectively.

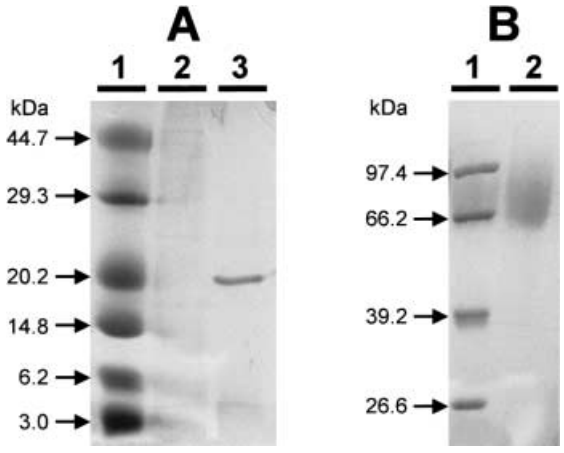

Fig. 6 Separation of the total extracellular protein fractions (50 µg) of **A** *A. niger* D15[pGT] (*lane 2*) and *A. niger* D15[*xyn2*] (*lane 3*) on 15% SDS-PAGE, and **B** *A. niger* D15[*egI*] (*lane 2*) on 10% SDS-PAGE. *Lane 1* Molecular weight markers with the sizes depicted on the *left*. The recombinant Xyn2 is present as a single protein species of about 21 kDa (**A**, *lane 3*) while the recombinant EgI is a more heterogeneous protein species of >60 kDa (**B**, *lane 2*)

Samples (2 mg) of the *A. niger* D15[*xyn2*] freeze-dried supernatant, corresponding to 50 µg of extracellular protein, were loaded onto a SDS-polyacrylamide (15%) gel (Fig. 6A). The Xyn2 protein was present as a single prominent band, while the rest of the native secreted proteins were barely visible. From the gel it was evident that the recombinant Xyn2 protein constituted a significant portion of the total amount of protein produced extracellularly by *A. niger* D15[*xyn2*]. The recombinant Xyn2 enzyme has a molecular mass of about 21 kDa, which corresponds to the size of the native Xyn2 produced by *T. reesei* (Lappalainen et al. 2000).

Samples of the *A. niger* D15[*egI*] freeze-dried supernatant (50 µg of unpurified extracellular protein) were loaded onto a SDS-polyacrylamide (10%) protein gel (Fig. 6B). The recombinant EgI was present as a heterogenous protein species, constituting a large portion of the extracellular protein fraction. The recombinant EgI exhibited a molecular mass varying from 62 to 100 kDa.

Discussion

cDNA copies of the *xyn2* and *egI* genes of *T. reesei* QM6a have been cloned and successfully expressed in the *A. niger* D15 strain. *A. niger* D15 is a nonacidifying pH mutant (*phmA*) derived from a protease-deficient (*prtT*) strain of AB1.13 (Gordon et al. 2000; Wiebe et al. 2001). Stable transformants were selected that constitutively expressed the *T. reesei* genes by means of the *gpd* promoter (Fig. 1). The recombinant strains *A. niger* D15[*xyn2*] and *A. niger* D15[*egI*] contained at least five and two copies of the *xyn2* and *egI* genes, respectively (Fig. 2A,B). However, not all the gene copies may be intact or functionally expressed. The integration site may also influence the level of gene expression from the indi-

vidual *xyn2* and *egI* gene copies (Verdoes et al. 1993, 1995).

The highest level of xylanase activity obtained from *A. niger* D15[*xyn2*] was 8,000 nkat/ml. This activity level compared extremely well with the 5,400 nkat/ml obtained from *T. reesei* Rut C-30 (Table 3) (Bailey et al. 1993a), when taking into account that the *T. reesei* activity represented the combined and synergistic action of the complete battery of xylanase enzymes, whereas the activity expressed in *A. niger* largely constituted that of the recombinant Xyn2 xylanase. The EgI endoglucanase activity obtained from *A. niger* D15[*egI*] was several fold higher than the levels obtained from other heterologous hosts (Table 3). *A. niger* is therefore the preferred host for heterologous expression of hydrolase genes, such as *xyn2* and *egI*.

The Xyn2 and EgI enzymes produced in different hosts had very similar biochemical characteristics (Tables 3). Our study indicated that recombinant EgI has a preference for CMC rather than lichenan, which was in contrast to the results obtained with expression of *egI* in *S. cerevisiae* by Penttilä et al. (1987). The recombinant Xyn2 and EgI constituted the bulk of the extracellular protein fraction produced by the recombinant *A. niger* D15[*xyn2*] and *A. niger* D15[*egI*] strains on molasses or glucose-based minimal (2xMM) media, respectively. The pH of the growth media was monitored throughout the study and never dropped below pH 4.5, thus preventing activation of the native *A. niger* extracellular acid proteases. SDS-PAGE did not reveal any visible degradation of the enzymes. The production of native extracellular proteins by *A. niger* D15[pGT] on molasses or 2xMM media was very limited, confirming that the recombinant Xyn2 and EgI proteins were produced at high homogeneity (Fig. 6).

The recombinant Xyn2 is similar in size to the native enzyme (Lappalainen et al. 2000). The EgI endoglucanase activity obtained was lower than what would be expected when considering the amount of protein present on the SDS-polyacrylamide gel (Fig. 6). This could imply that a significant amount of protein was inactive, as was reported by Aho et al. (1996), who found that only 2% of the heterologously produced EgI was active. The endoglucanase protein was also more heterogenous in size when expressed in *A. niger* than when expressed in *S. cerevisiae* (Penttilä et al. 1987). This phenomena, however, is not uncommon with expression of *T. reesei* enzymes in *Aspergillus* and can be ascribed to variable glycosylation patterns (Takashima et al. 1998). Glycosylation, responsible for the heterogenous nature of the protein, could be responsible for the lack of activity, with the sugars covering the active site.

The use of the *gpd* promoter enabled the strain to produce the recombinant enzymes upon germination without the need for an inducer, simplifying the production of recombinant enzymes in bulk. The particular strain used was a pH mutant and therefore did not significantly acidify the growth media, as is often observed for *A. niger* strains. Therefore, this strain does not produce high amounts of acidic proteases that could extracellularly degrade the recombinant proteins produced. The use of a pH mutant of *A. niger* as host thus led to the production of a significant amount of extracellular protein. The control strain, *A. niger* D15[pGT], exhibited low levels of endoglucanase and xylanase activity when cultured on molasses or 2xMM media, despite the fact that *Aspergillus* spp. are known for their potent cellulase and xylanase activities. The levels of native *Aspergillus* glycoside hydrolases remained insignificantly low even after depletion of the glucose in the media.

In general, the levels of recombinant protein production could further be increased by increasing the inoculum concentration or by using salt adaptation. Redkar et al. (1998) found that the secretion capacity of the fungus is directly related to the number of hyphal tips. Increased hyphal branching or salt adaptation (as a result of osmotic stress) could increase the production capacity of recombinant *Aspergillus* heterologously expressing glycoside hydrolases.

The ability of *A. niger* to grow on inexpensive industrial waste such as molasses makes it ideal for waste control, as molasses is a waste product of the sugar industry. It has the ability to support the growth of *A. niger* D15 without the requirement of additional nutrients, despite the fact that this *pyrF* strain requires uridine in the growth media. The high levels of enzyme production obtained, as observed for recombinant Xyn2 and EgI, in shake-flask cultures and the ease of cultivation make it worthwhile to consider the use of *A. niger* for industrial enzyme production on molasses as a commercially available carbon source.

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